A Virus BioResistor (VBR) for the Detection of the Bladder Cancer Marker DJ-1 in Urine at 10 pM in One Minute

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1. Materials and Fabrication of VBR

Additional details relating to this process outlined in the Experimental Section are the following: Gold-film electrodes were cleaned by O_2 plasma for 10 min immediately before use. Scotch tape was placed on the ends of the electrodes to protect the contacts. To obtain low DC resistance PEDOT-PSS films, 3% (v/v) ethylene glycol was mixed with PEDOT-PSS at 550 rpm for 30 min. To obtain high DC resistance PEDOT-PSS films, 1.5% (v/v) ethylene glycol was mixed with PEDOT-PSS at 550 rpm for 30 minutes. The mixture was spin-coated on the gold electrodes at 2500 rpm, 80 s and baked for 1 h at 90 °C. Electrodes were then allowed to equilibrate at room temperature and the cell was then mounted on the gold-film electrodes followed by the incubation of the electrodes in PBS for 30 min. Next, virus-PEDOT films were electropolymerized onto the PEDOT-PSS/gold-film electrodes using a platinum foil counter and a mercurous sulfate electrode (MSE). Virus-PEDOT films were prepared by cycling between 0.2 V and 0.8 V at a scan rate of 20 mV/s in plating solution using a PARSTAT 2263 potentiostat controlled by Electrochemistry PowerSuit 2.6 software. Plating solutions contained 8 nM M13 bacteriophage, 12.5 mM $LiClO_4$, 2.5 mM EDOT, and were electropolymerized for 2 cycles.

2. Process windows compliance assessment of VBRs.

*VBR*s were evaluated at every step of the fabrication process to ensure the reproducibility of signal at each DJ-1 concentration. Starting with the fabrication by photolithography of gold electrodes, the *VBR* is prepared in five steps (Figure S1). The parameters measured at each of these steps is indicated in the diagram for Fig. S1. In this diagram, the following definitions apply: R_{au} is the dc resistance of the gold electrons prepared in step 1, measured along their longest dimension, $R_{PEDOT\text{-}PSS}$ is the dc resistance of the PEDOT-PSS film produced in step 2, Z_{im} and Z_{re} are the baseline impedances measured for the PEDOT-PSS film after incubation in PBS for 30 min in step 3; i_p is the peak current for the electropolymerization, by cyclic voltammetry, of the virus-PEDOT composite in step 4. The *VBR* device yield using the process windows described below was ≈60%.

Step 1: The DC resistance between two end-points on the individual gold pad should be 3.9 to 5 Ω. This resistance adds to the resistance of spin-coated PEDOT-PSS resistance to yield the final DC resistance across PEDOT-PSS coated electrodes.

Step 2: Chips with DC resistance across the baked PEDOT-PSS films of 240 to 380 Ω are then used for further fabrication.

Step 3: To eliminate the baseline drift, the PEDOT-PSS films from the previous step are immersed in PBS for 30 minutes. This step results in increased resistance of the swollen PEDOT-PSS film, tracked by the Nyquist Zre = 300 – 500 Ω and Zim = 80 – 150 Ω values.

Step 4: The cyclic voltammogram is indicative of EDOT polymerization quality and phage entrapment. The shape of the voltammogram along with the anodic peak current (i_p) values are important screening parameters. The i_p separation between two subsequent scans is approximately 1×10^{-4} A.

Step 5: Visual inspection of the newly formed biorecognition layer for any abrasions, prevents unusual Nyquist drift and non-specific signals.

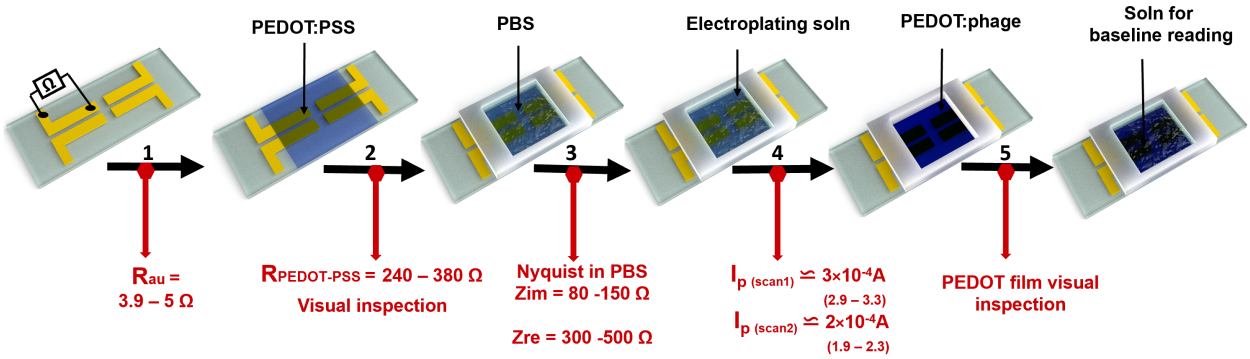


Figure S1 – Process flow for the VBR fabrication process, including the process window parameters that were enforced for this process, indicated in red.

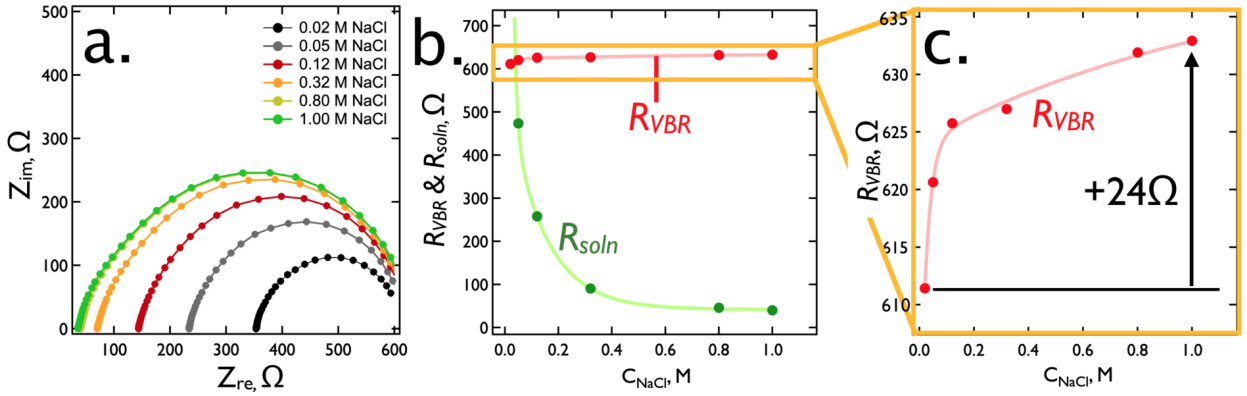


Figure S2. – Influence of C_{NaCl} on *VBR* response in the absence of protein. a). Nyquist plots for a single VBR in six aqueous NaCl solutions ranging in concentration from 0.02 M to 1.0 M, as indicated. b). Plot of R_{VBR} and R_{soln} as a function of C_{NaCl}. R_{soln} (green trace) decreases in proportion to $1/C_{NaCl}$ qualitatively as expected, but R_{VBR} is weakly affected, increasing by just 24 Ω against a background of ≈ 600 Ω (b,c).

Table S1. *VBR* circuit elemental values corresponding to the Nyquist plots of Figure 8a-c.

Fig 8a	**Synthetic Urine**			**10 pM DJ-1**	
	Value	Stdev		Value	Stdev
R_{sol} (Ω)	338.5	0.2		333.6	0.7
R1 (Ω)	1097	4		1136	8
C_{VBR} (F)	1.040×10^{-5}	4×10^{-8}		1.010×10^{-5}	5×10^{-8}
R_{VBR} (Ω)	1617	2		1674	2
CPE, Q (F)	2.570×10^{-5}	5×10^{-8}		2.54×10^{-5}	2×10^{-7}
CPE, n	0.85	0.00		0.85	0.00
Fig 8b	**Synthetic Urine**			**1 nM DJ-1**	
	Value	Stdev		Value	Stdev
R_{sol} (Ω)	331.7	0.4		333.6	0.1
R1 (Ω)	1490	2		1823	6
C_{VBR} (F)	8.70×10^{-6}	2×10^{-8}		8.610×10^{-6}	8×10^{-9}
R_{VBR} (Ω)	1663.3	0.4		2010	10
CPE, Q (F)	2.380×10^{-5}	9×10^{-8}		2.53×10^{-5}	3×10^{-7}
CPE, n	0.86	0.00		0.86	0.00
Fig 8c	**Synthetic Urine**			**300 nM DJ-1**	
	Value	Stdev		Value	Stdev
R_{sol} (Ω)	304.5	0.1		275	1
R1 (Ω)	2550	20		4110	50
C_{VBR} (F)	8.49×10^{-6}	5×10^{-8}		6.99×10^{-6}	9×10^{-8}
R_{VBR} (Ω)	1983	5		2634	3
CPE, Q (F)	2.66×10^{-5}	1×10^{-7}		2.78×10^{-5}	2×10^{-7}
CPE, n	0.86	0.00		0.86	0.00

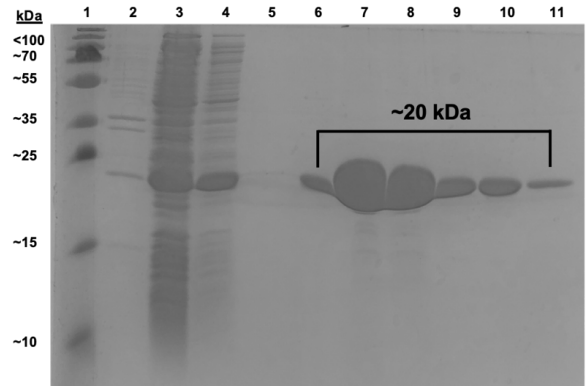
3. Protein expression and selection of DJ-1 binders

3.1. Expression and purification of the bladder cancer biomarker and protein deglycase DJ-1.

The plasmid pET3a-His-DJ1 was heat shock-transformed into BL21(DE3) *E. coli* cells and incubated overnight on an agar plate supplemented with carbenicillin (50 µg/mL). A single colony was selected and inoculated into 20 mL of LB media supplemented with carbenicillin (50 µg/mL) before incubation overnight at 37 °C with shaking at 225 rpm. A 5 mL aliquot of the overnight culture was transferred to 500 mL of LB media supplemented with carbenicillin (50 µg/mL) and shaken at 225 rpm and 37 °C until OD_{600} reached 0.6. The culture was induced through addition of IPTG (1 mM), and was incubated at 30 °C with shaking at 225 rpm for 4 h.

Following bacterial overexpression, the culture was centrifuged at 6 krpm (4302 x g) for 30 min at 4 °C, and the supernatant was discarded. The resulting pellet was resuspended in 25 mL of lysis buffer (1X TBS, 10 mM 2-mercaptoethanol (BME), 1X Halt™ protease inhibitor cocktail, pH 7.5) and lysed by sonication. This lysed cell solution was centrifuged at 10 krpm (11952 x g) for 1 h at 4 °C. The protein was purified from the supernatant by immobilized metal affinity chromatography (IMAC) with Ni^{2+}-NTA resin (BioRad). The resultant eluted fractions were visualized by 12% acrylamide SDS-PAGE and ImageJ analysis (Figure S3). The appropriate eluted fractions were combined and stored at -20 °C in storage buffer (1X TBS, 10 mM BME, 50% glycerol, pH 7.5). DJ-1 enzyme activity was assayed by hemithioacetal consumption.[2] Briefly, methylglyoxal (7.5 mM) and L-glutathione (7.5 mM) were mixed and pre-incubated for 30 min at room temperature in sodium phosphate (50 mM, pH 7.0) to form hemithioacetals. DJ-1 (1 µM), diluted in sodium phosphate (50 mM, pH 7.0), was then added to the reaction mixture. Changes in hemithioacetal levels were monitored over 90 min as a time-dependent change in the characteristic absorbance at 288 nm.

a.



Lane	Sample
1	PageRuler Plus Prestained Protein Ladder
2	*E. coli* cell pellet
3	Cell lysate
4	Wash 1
5	Wash 2
6	Elution 1
7	Elution 2
8	Elution 3
9	Elution 4
10	Elution 5

b.

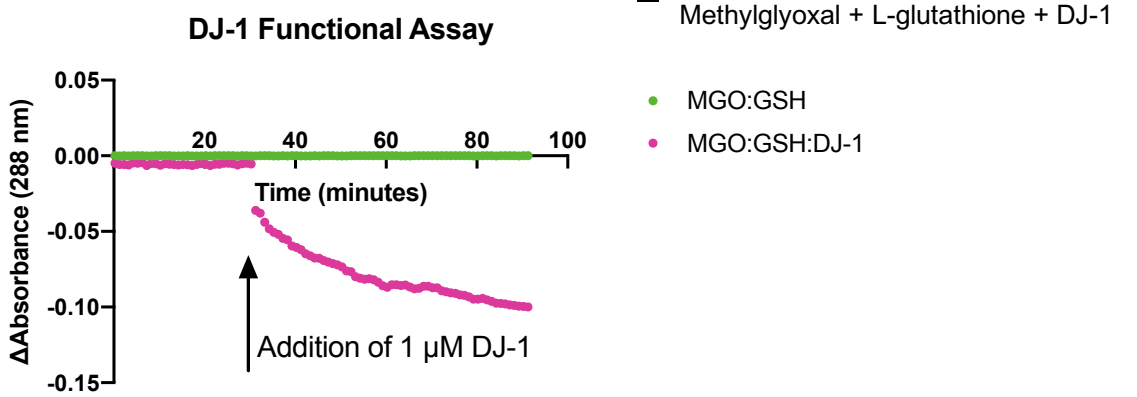


Figure S3. a). SDS-PAGE analysis of purified DJ-1 after IMAC purification. ImageJ analysis of similar gels quantified DJ-1 purity as >99% post-purification. b). DJ-1 enzyme activity assay. DJ-1 (1 μM) was added to methylglyoxal (7.5 mM) and L-glutathione (7.5 mM). The decrease in absorbance at 268 nm over 90 min compared to the negative control indicates changes in hemithioacetal levels and demonstrates the expected DJ-1 enzyme activity.

3.2. Selection of DJ-1 ligands. Four rounds of phage-based selections identified four DJ-1 binding phage. In each round, 15 wells of a 96-well Nunc MaxiSorp microtiter plate were coated with DJ-1 (10 μg/mL) in PBS (pH 8.0, 100 μL per well) and incubated overnight at 4 °C with shaking at 150 rpm. This solution was discarded and a blocking solution containing 0.2% (w/v) nonfat milk in PBS (400 μL per well) were added to the wells, and the plate was incubated at room temperature at 150 rpm for 30 min. In sequential rounds, this blocking step was performed with hen egg white lysozyme, ovalbumin, or a mixture of BSA and HSA to minimize selection of peptide ligands to blocking agents.

After washing three times with wash buffer (0.05% (v/v) TWEEN 20 in PBS, 300 µL per well), mega random peptide libraries (MRPLs)[3] were diluted to a final concentration of 60 nM in binding buffer (0.2% (w/v) BSA, 0.05% (v/v) TWEEN 20 in PBS). The diluted libraries were added to the microtiter plate (100 µL per well) before incubation for 90 min with shaking at 150 rpm at room temperature. Next, the wells were washed to remove non-specific phage ligands. In each round of selections, the numbers of washes were increased by three to a maximum of 15.

The bound phage were eluted from the plate by adding 0.1 M HCl (100 µL per well) and sonicating the plate in a water bath for 10 min. The eluted phage solution was immediately neutralized by transferring the solution to 1/3 volume of Tris-HCl (1 M, pH 8.0). A portion of the eluted phage solution was used to infect a 20 mL LB culture (supplemented with 5 µg/mL tetracycline) of log phage *E. coli* XL1 Blue cells. The cells were incubated with shaking at 225 rpm at 37 °C for 1 h. Next, the culture was further infected with M13KO7 helper phage (NEB) to achieve a multiplicity of infection of 4.6. After 45 min of incubation at 37 °C with shaking at 225 rpm, the culture was transferred to 200 mL of 2YT (16 g tryptone, 5 g NaCl, 10 g yeast extract in 1 L autoclaved water) supplemented with 50 µg/mL carbenicillin and 20 µg/mL kanamycin and incubated at 225 rpm at 37 °C for 16 to18 h.

The cultures were centrifuged at 10 krpm (15300 x g) for 10 min. The supernatant was decanted into a centrifuge tube containing 1/5 the volume of PEG-8000 (20%, w/v) and NaCl (2.5 M). The tube was inverted 5 times and stored on ice for 30 min followed by an additional centrifugation at 10 krpm (15300 x g) for 15 min. The supernatant was decanted, and tubes were centrifuged for an additional 4 min at 4 krpm (2429 x g). The pellets were resuspended in PBS and the precipitation steps were repeated. Phage concentrations were quantified by measuring absorbance at 268 nm. Finally, the phage were diluted to 60 nM, flash frozen with glycerol (10%, v/v), and stored at -80 °C.

After four rounds of selections, spot assays were performed on 96 selectants. Briefly, individual phage colonies were amplified in 96 deep well plates as before. After centrifugation at 3 krpm (1462 x g), the supernatants were assayed by phage-based ELISA (see *Phage ELISA* method) to assess binding to either DJ-1 or the blocking agent, casein. From these screens, four unique potential DJ-1 ligands were isolated and identified by Sanger sequencing. The peptides' specificity for DJ-1 was tested by additional screening for binding to a panel of proteins including BSA, HSA, ovalbumin, lysozyme, and *E. coli* supernatant. Only two of the four potential ligands showed specificity for DJ-1 (Table S2). Ultimately, only one ligand was incorporated into the sensor design due to its significantly stronger apparent binding affinity than the other ligand, as measured by ELISA. This ligand is referred to as DJ-1 ligand one (DL1).

Table S2. DJ-1 binding ligand peptide sequences isolated by phage display selections.

Ligand	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
DL1	K	Y	R	Y	V	C	H	D	V	G	G	T	L	Y	C	I	R	D	*	V
DL2	R	P	T	L	Q	E	L	C	*	T	I	Y	V	C	Y	F	V	D	L	G

Asterisk (*) indicates TAG Amber stop codon.

3.3. Site-directed mutagenesis of DL1. The phage displayed DL1 ligand included an amber stop codon (TAG). In an amber suppressor strain of *E. coli* such as XL1-Blue, glutamine will be incorporated at a low rate of occurrence.[4] To reduce this inherent heterogeneity, Q5 Site-Directed Mutagenesis (NEB) was performed to replace the TAG stop codon with CAG, which encodes for glutamine. Sanger sequencing confirmed the successful introduction of the glutamine codon.

3.4. Phage propagation and purification. The phagemid DNA was transformed into SS320 competent *E. coli*, and transformants were plated on a carbenicillin-supplemented (50 µg/mL) agar plate before incubation at 37 °C overnight. A single colony was selected to inoculate 25 mL of 2YT supplemented with carbenecillin (50 µg/mL) and tetracycline (2.5 µg/mL). The culture was shaken at 37 °C until OD_{600} reached 0.5; then, 30 µM IPTG and sufficient M13KO7 to achieve a multiplicity of infection of 4.6 was added. After an additional 45 min incubation, 8 mL of the culture was used to inoculate a 150 mL of 2YT supplemented with carbenecillin (50 µg/mL), kanamycin (20 µg/mL), and IPTG (30 µM). This culture was incubated at 30 °C with shaking at 225 rpm for 18 h.

The phage were precipitated as described above, and the resulting phage pellets were resuspended in 1X PBS with TWEEN 20 (0.05%, v/v) and glycerol (10%, v/v), separated into 1 mL aliquots, flash frozen with liquid nitrogen, and stored at -80 °C. To prepare for devices or ELISAs, the phage solution was thawed on ice, precipitated a second time, and diluted to 40 nM in either $LiClO_4$ (12.5 mM) or PBS, respectively.

4. **Phage ELISA.** To characterize the apparent binding affinity of the selected phage, 5 µg/mL of DJ-1 in Na_2CO_3 (50 mM, pH 9.6, 100 µL per well) were added to a 96-well Nunc MaxiSorp microtiter plate. The plate was incubated at 4 °C with shaking at 225 overnight. Next day, the solution was discarded and a blocking solution of BSA (0.2%, w/v) in PBS (400 µL per well) was added to the coated wells. The plate was next incubated at room temperature for 30 min with shaking at 150 rpm. The coated

wells were washed three times with wash buffer (300 µL per well), followed by the addition of either DL1 or negative control Stop4 phage serially diluted in binding buffer (100 µL per well). The plate was incubated for 60 min at room temperature and shaking at 150 rpm. Next, the plate was washed three times. Finally, a 1:5000 dilution of HRP/anti-M13 monoclonal conjugate (GE Healthcare Life Sciences, 100 µL per well) was added, and the plate was incubated at room temperature with shaking at 150 rpm for 30 min. After five additional washes with wash buffer and one with PBS, 1-Step™ Ultra TMB-ELISA Substrate Solution (ThermoScientific, 100 µL per well) were added to each well. After 5 min, H_2SO_4 (2 M, 100 µL) was added to the wells and the absorbance at 450 nm was measured with an Epoch Microplate Spectrophotometer (BioTek). Data were analyzed with GraphPad Prism 8 and fit with a four-parameter logistic curve fit. The apparent dissociation constant ($K_{d,\,app}$) for the interaction between the phage-displayed DL1 and DJ-1 was calculated to be 14 pM.

5. **DJ-1 phage-antibody sandwich ELISA.** To simulate the DJ-1/DL1 interaction in the VBR format, DL1 (1 nM) and Stop4 (1 nM) phages were bound to a 96-well microtiter plate as described above. The plate was treated identically to the indirect phage ELISA until the first binding step; DJ-1 dilutions were prepared and 100 µL of the diluted proteins were added to every well. The plate was incubated for 60 min at room temperature with shaking at 150 rpm. After washing three times with wash buffer, the primary antibody, PARK7/DJ-1 Antibody (LifeSpan Biosciences, Inc.), was diluted to 1:1000 in binding buffer and 100 µL were added to each well. The plate was incubated again for 60 min at room temperature with shaking at 150 rpm, and washed three times. A secondary antibody, anti-rabbit IgG (Sigma-Aldrich), was diluted to 1:5000 in binding buffer and 100 µL were added to each well. The plate was incubated for 30 min and washed five times with wash buffer and once with PBS. Finally, the HRP activity was detected as described above and the $K_{d,\,app}$ was determined to be 206 nM.

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